Evidence for a Circadian Effect on the Reduction of Human Growth Hormone Gene Expression in Response to Excess Caloric Intake*

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Rhythmicity of biological functions is fundamental for optimal adaptations to environmental cues. Growth hormone (GH) is a major metabolic homeostatic factor that is secreted with a circadian pattern, but whether it is synthesized rhythmically is unknown. We used transgenic mice containing the human (h) GH gene (*hGH1*) locus to investigate the rhythmicity of hGH synthesis and secretion and to show that RNA and secreted protein levels oscillate over a 24-h cycle. Analysis of *hGH1* promoter sequences revealed an enhancer motif (E-box) element that binds the circadian transcriptional machinery (Bmal1 and Clock). Furthermore, Bmal1/Clock were able to transactivate the *hGH1* promoter, and mutation of this E-box element adversely affected basal activity after gene transfer. The ability of Bmal1 to bind the *hGH1* promoter region containing the E-box element was confirmed in the *hGH1* transgenic mouse pituitary *in situ*. Occupancy was reduced in mice fed a high fat diet during the light (inactive) stage of the daily cycle in mice and corresponded to a decrease in *hGH1* RNA levels. The decreases in occupancy and RNA levels were not seen, however, during the dark (active) stage. A chromatin loop required for efficient postnatal *hGH1* expression was negatively affected by the high fat diet in the light but not dark stage similar to the pattern observed with Bmal1 association with the promoter region. This is the first evidence that hGH synthesis follows a diurnal rhythm and of dynamic associations of the circadian machinery with a component of a chromosomal structure of the *hGH1* locus that is essential for efficient expression.

Rhythmicity of biological function including the endocrine system is fundamental for optimal adaptation to environmental cues (1, 2). Human (h)[2] growth hormone (GH) secretion is subject to a circadian and diurnal rhythm that follows a sleep pattern (3, 4). Specifically, the peak in hGH secretion occurs during sleep in the dark stage of the daily cycle (3, 4). If this sleep pattern is disrupted, however, compensatory and largely unpre-

dictable pulses of GH release are observed during the light/ awake stage, as demonstrated in shift workers (5, 6). This suggests that GH production is not solely under the control of the sleep/awake cycle but is regulated at a higher level of complexity and perhaps by the circadian machinery. However, it is not known whether hGH synthesis occurs, like secretion, with a circadian rhythm (7). The difficulty in establishing that hGH synthesis, and specifically the hGH gene (*hGH1*), is a target for circadian rhythm, is because of a lack of access to premortem human pituitary samples. Animal models are also not optimal. There are distinct differences in structure between human (primate) and murine (non-primate) GH genes, including in both the flanking and coding DNA. As a result, hGH and mouse (m) GH have the potential for different regulatory control and function (8 –14).

In this study, we used 171hGH/CS transgenic (TG) mice that preferentially express *hGH1* in mouse pituitary somatotrophs (15, 16). This pattern of tissue/cell-specific expression is a result of including the intact hGH gene and locus control region (LCR) in a ~150-kb fragment of human chromosome 17 (15) as part of the transgene (16). The LCR can act as a tissue- or cell-specific enhancer and also confer appropriate site of integration-independent expression of a transgene (15–18). These mice grow normally and specifically express (not overexpress) *hGH1* in somatotrophs of the anterior pituitary gland (15, 16). This property depends on the presence of essential hypersensitive site (HS) regions I–III and V within the LCR. HS I/II is seen in human pituitary somatotrophs, is re-established in the *hGH1* TG mouse genome, and is the major determinant of pituitary *hGH1* expression (13, 19 –22). Physical interaction between HS I/II and the local *hGH1* promoter and looping of intervening (14.5 kb) chromatin (23–25) is essential for *hGH1* activation (13, 24) and postnatal expression (23, 26).

Microarray studies of transcripts have revealed that 10 –30% of the human genome is under the control of circadian molecular clocks (27). Daily rhythms seen in these transcripts are controlled by an endogenous circadian timing system (28, 29). These self-directed rhythms are generated by a well characterized intracellular transcriptional feedback loop involving a *cis*-regulatory element known as an enhancer box (E-box) (28, 29). The core players of this molecular transcriptional feedback system, which orchestrates the cyclic expression of clock-controlled genes, are Clock (circadian locomotor output cycles kaput), Bmal1 (brain and muscle ARNT-like 1), Period proteins Per1–3, two Cry (Cryptochrome) proteins, and Rev-erbα

[2] The abbreviations used are: h, human; GH, growth hormone; qPCR, quantitative PCR; TG, transgenic; HFD, high fat diet; m, mouse; LCR, locus control region; HS, hypersensitive site; ZT, zeitgeber time; CD, chow (control) diet; 3C, chromosome conformation capture.

(28–30). This sophisticated and elegant transcriptional clock machinery ensures coordinated and orchestrated molecular and physiological responses by affecting target gene expression with changes in the environment, for example, through changes in diet (31).

hGH levels are known to fluctuate dynamically under different physiological and pathophysiological conditions, including excess caloric intake and obesity. Normal healthy individuals that indulge in short term overeating display a dramatic suppression of serum GH levels. Interestingly, this phenomenon happens when obesity is not an issue (32). We have previously shown that hGH production is extremely sensitive to increased caloric intake within the continuum of obesity progression. Three days of excess caloric intake induced by high fat diet consumption led to a 75% reduction in *hGH1* RNA levels and impaired secretion in transgenic mice containing the hGH gene and LCR (33). Unlike *hGH1*, the endogenous mGH gene (*mGh*) displayed no negative response to the hyperinsulinemic conditions induced by acute excess caloric in transgenic and wild type CD1 mice *in vivo* (33). An explanation for this differential response could relate to the presence of an E-box DNA element in the proximal *hGH1* promoter region that is absent from equivalent *mGh* promoter DNA (34). Furthermore, the presence of this E-box element offers a potential target for Bmal1 and Clock proteins.

Here, we provide evidence that hGH synthesis and specifically *hGH1* expression are under the control of a circadian rhythm and a target of Bmal1/Clock interaction. Furthermore, we show that excess caloric intake can perturb *hGH1* Bmal1/E-box DNA element binding, as well as the chromatin loop linking HS I/II and promoter regions. These observations are presented and discussed in the context of the daily light/dark cycle.

Experimental Procedures

Animals and Diet—All procedures involving animals, their tissues, and their cells conform to the Guide for the Care and Use of Laboratory Animals published by the Canadian Council on Animal Care and were approved by the Animal Protocol Management and Review Committee at the University of Manitoba. Animals were housed with *ad libitum* access to food and water in an environmentally controlled room maintained on a 12-h light/dark cycle with lights on at zeitgeber time (ZT 0; which refers to the number of hours after the light phase onset of 6 a.m. and lights off at ZT 12 or 6 p.m.). Control regular chow diet (CD; fat, 26 kcal%; carbohydrate, 54 kcal%; protein, 20 kcal% from Prolab-RHM 3000) or high fat diet (HFD; fat, 60 kcal%; carbohydrate, 20 kcal%; protein, 20 kcal% from Research Diets, New Brunswick, NJ) was supplied as palatable pellets for 3 days to 4-week-old male mice (16, 34). The mice were maintained on their respective diets until time of euthanization (cervical dislocation) and assay.

Blood Chemistry—Trunk blood was collected and allowed to clot at 4 °C for 15 min, and serum was separated by centrifugation (9300 × *g* for 5 min). Serum samples were stored at −80 °C until assessed by human GH ELISA (22-HGHHUU-E01, ALPCO).

TABLE 1
Primers used for qPCR

Transcript		Primer sequence
hGH1	Forward:	CCTAGAGGAAGGCATCCAAA
	Reverse:	GCAGCCCGTAGTTCTTGAGTAG
mGH	Forward:	ACGCGCTGCTCAAAAACTAT
	Reverse:	CACAGGAGAGTGCAGCAGAG
mClock	Forward:	ACAGTTTCACGAGGGTGGTC
	Reverse:	TCCCTACCGTCTCATCAAGG
mBmal1	Forward:	CCACCTCAGAGCCATTGATACA
	Reverse:	GAGCAGGTTTAGTTCCACTTTGTCT
mPer2	Forward:	TGTGCGATGATGATTCGTGA
	Reverse:	GGTGAAGGTACGTTTGGTTTGC
mRev-erbα	Forward:	AGACTTCCCGCTTCACCAAG
	Reverse:	AGCTTCTCGGAATGCATGTT
mCry1	Forward:	TGAGGCAAGCAGACTGAATATTG
	Reverse:	CCTCTGTACCGGGAAAGCTG
mCry2	Forward:	CTGGCGAGAAGGTAGAGTGG
	Reverse:	GACGCAGAATTAGCCTTTGC
mC-fos	Forward:	GACAGCCTTTCCTACTACCATTCC
	Reverse:	GGACAGATCTGCGCAAAAGTC

RNA Preparation and Quantitative Real Time Reverse Transcriptase-PCR—Total RNA was isolated using QIA shredder and RNeasy Plus mini kit (Qiagen; 79656, 74136). The quality of the extracted RNA was assessed by agarose gel electrophoresis. Total RNA (1 μg) was transformed to cDNA using the QuantiTect reverse transcription kit (Qiagen; 205314) according to the manufacturer's instructions. Quantitative real time RT-PCR (qPCR) analyses were done in a 7500 system (AB Applied Biosystems, Warrington, UK) with specific primers (Table 1). Reactions (20 μl) included 10 μl of Power SYBR green Master mix (Applied Biosystems), 1 μl of forward and reverse primers (0.5 pM), and 0.1 μg of cDNA. Thermal cycling was started with 5 min of denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 s, annealing at 60 °C for 15 s, and 72 °C for 30 s. Minus RT controls were done using the same PCR primers and conditions as a control for genomic DNA contamination. Specific amplifications were identified by a single peak in the melting curve and a single band in the final PCR product visualized on 1% agarose gel. The gene expression level in each sample was calculated from a standard curve (for each primer set) and normalized to mouse β-actin expression. Tests were run at least in duplicate and on three mice for each experiment.

Cell Culture, Plasmid Constructs, and Gene Transfer—HEK293 cells were maintained in a monolayer culture in DMEM (Invitrogen; 12100-061) supplemented with 0.5 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and FBS at an appropriate (v/v) percentage in a humidified air/CO_2 (19:1) atmosphere, at 37 °C (35). The cells were harvested using trypsin-EDTA after a single wash with PBS.

The 0.5 kb of the mGh promoter region was generated by PCR using primers (forward: 5′-GATCGGGATCCTCCCCA-AAAGTTATCTTCT-3′, reverse: 5′-CGATCAAGCTTGGA-ATCTGGACTCTAGGATG-3′) that introduce BamHI (5′-GGATCC-3′) and HindIII (5′-AAGCTT-3′) sites for subcloning into the BamHI and HindIII sites of the firefly luciferase vector, pXP1-Luc (36). Mutation of the WT E-box element from 5′-C<u>CACGTG</u>ACC-3′ to 5′-C<u>CAgcTG</u>ACC-3′ in the 0.5-kb *hGH1* promoter region, was done using a two-step PCR approach and use of specific primers as described previously (37). In brief, a double-stranded fragment with a 21-nucleotide overlap of the region to be mutated was generated, and

BamHI and HindIII sites were then introduced for subcloning into pXP1-Luc. The cDNA expression vectors for mouse Clock, previously reported as Gal4-Clock/UA256, and Bmal1, previously reported as Gal4-Bmal1/UA255, were kindly provided by Dr. Urs Albrecht (University of Fribourg, Fribourg, Switzerland) (38). The construct for human period circadian protein homolog 2 (Per2) luciferase construct (pGL3-hPer2-Luc) was kindly provided by Dr. Louis Ptacek (University of California, San Francisco) (39). Trans-IT293 reagent (Mirus Biol Corp., Madison, WI) diluted with serum-free DMEM and containing expression vectors or hybrid reporter genes was used for transient transfection of HEK293 cells. For expression of cDNA vectors, up to 4 μg of plasmid DNA (expression vector) was used per 1×10^6 cells/100-mm plate. For transfection with reporter genes, up to 0.5 μg of reporter gene plasmid and 10 ng of pRL-TKp-Luc plasmid (as a control for DNA uptake) was used per 2.5×10^5 cells/well (6-well plate). The cells were treated with DNA/reagent for 24 h, washed with PBS, and harvested, and luciferase assays were done 48 h later.

Nuclear Protein Extraction and Detection—The EpiSeeker nuclear extraction kit was used according to the manufacturer's instructions (Abcam; ab113474) (40). Briefly, the cells were suspended in hypotonic buffer containing dithiothreitol and proteinase inhibitors on ice for 15 min. Nuclei were separated by centrifugation (14,000 $\times$ g for 1 min at 4 °C), and the nuclear pellet were extracted in a hypertonic lysis buffer for 30 min on ice. Following centrifugation (14,000 $\times$ g for 10 min at 4 °C), the supernatant containing the nuclear fraction was collected, and protein concentration was determined by a Bradford protein assay (Bio-Rad).

For detection of Clock and Bmal1, 20 μg of nuclear and cytoplasmic proteins from HEK293 cells transfected with expression vectors were separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride membrane, and immunoblotted with anti-Clock (Abcam; ab3517) and Bmal1 (Abcam; ab3350) antibodies. β-Tubulin (Santa Cruz; sc-9104) and lamin B (Santa Cruz; sc-6217) were assessed as controls for protein loading for whole cell lysates and nuclear proteins, respectively. The proteins were visualized using horseradish peroxidase-conjugated anti-IgG secondary antibody and ECL plus immunoblotting detection reagents (Thermo Fisher Scientific).

Electrophoretic Mobility Shift Assay—EMSA and competition with oligonucleotides was performed essentially as previously described (41). Oligonucleotides (Invitrogen) were radiolabeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs). Briefly, 4 μg of HEK293 nuclear extract was incubated with EMSA buffer containing 2 μg of poly(dI-dC) for 5 min. Radiolabeled oligonucleotide probe (1 ng) was then added, and the reactions were incubated for a further 10 min at room temperature. For competition, 50-fold molar excess of unlabeled oligonucleotide was added during the preincubation period. The DNA-protein complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels and visualized by autoradiography.

Luciferase Assay—Luciferase activity was measured using the Dual-Luciferase assay system (Promega; PRE1960), with a photon counting luminometer (LUMAT LB9507; EG&G Berthold). In brief, cell pellets were resuspended in 50 μl of lysis buffer

TABLE 2
Primers used for ChIP-qPCR

Genomic regions	Primer sequence
hGH1 promoter	Forward: CCCCTTCTCTCCCACTGTTG Reverse: AACCCTCACAACACTGGTGAC
Untr6	Forward: TCAGGCATGAACCACCATAC Reverse: AACATCCACACGTCCAGTGA

(100 mM Tris-HCl, pH 7.8, 0.1% Triton X-100) for 10 min on ice and centrifuged at 15,800 $\times$ g for 15 min at 4 °C. The values were normalized through co-transfection with pRL-TKp-Luc and assessment of *Renilla* luciferase in a dual assay as well as with protein concentration, which was assessed using the Bradford protein assay.

ChIP Assay—ChIP assay was done with isolated mouse anterior pituitaries using a EZ-Magna ChIP kit (17-10086; Millipore) as reported previously (33). In brief, pituitaries were cross-linked with 1% of formaldehyde at room temperature for 30 min before lysis. Chromatin was fragmented by sonication (100 s in 10-s pulses and 100% amplitude) using a Q800R sonicator (Qsonica, Newtown, CT). Insoluble material was removed by centrifugation. DNA content was then measured by spectrophotometry. Soluble chromatin was immunoprecipitated with 5 μg of specific antibodies (indicated in figure legends) along with magnetic protein (A/G) beads overnight with rotation at 4 °C. The eluted chromatin separated from magnetic beads was reverse cross-linked at 65 °C overnight, and DNA was isolated using the QIAquick PCR purification kit (Qiagen). Quantitative PCR was done under conditions standardized for each primer set (Table 2). Each qPCR was carried out in duplicate in a 20-μl reaction volume by using 5 μl of the 1% of input DNA and 5 μl of preamplified (15 cycles) eluted immunoprecipitated DNA and 10 μl of Power SYBR green Master mix (Applied Biosystems). Dissociation curves were analyzed as a mean to ensure the quality of amplicons and to monitor primer dimers. Final PCR products were visualized as a single band on 1% agarose gel. ChIP enrichment was determined based on a percentage input method (42). Briefly, the signals obtained from the immunoprecipitated DNA amplification were divided by the signals obtained from an input sample. Enrichment was calculated based on the formula 100 $\times$ 2 (CT adjusted input − CT Enriched), and data are presented as percentages of input and relative fold change compared with the control, which is arbitrarily set to 1.

Chromosome Conformation Capture (3C) Assay—The 3C assay was performed essentially as described (33, 43, 44). Briefly, mouse pituitaries (four pituitary glands/3C test) were homogenized in a tissue nuclei isolation buffer to obtain a cell suspension. The cells were cross-linked for 10 min at room temperature using 1% formaldehyde-HEPES buffer, and the reaction was quenched by addition of 0.125 M glycine. The cells were washed with PBS and lysed in 1 ml of ice-cold lysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 0.2% Nonidet P-40) for 30 min at 37 °C. Triton X-100 was added to obtain a 1% final concentration, and then the nuclei were incubated for a further 30 min at 37 °C. The nuclei were washed with restriction enzyme (BglII) digestion buffer, and then cross-linked DNA was digested overnight with BglII. The restriction enzyme was inac-

TABLE 3
Primers used for the 3C assay

Genomic regions	Primer sequence
Nested PCR for HS I/II-hGH1p	HS I/II: ACAGCACCCATGAGGCAACA hGH1p: CAGGATAGCCAGTCCTTGAGAC
qPCR for HS I/II-hGH1p	HS I/II: CCATTCTTAACCTCTCAGTGACCA hGH1p: GAAAAAGAAAAAGAAAGATGCCCTGT

tivated with 0.1% SDS and incubating at 65 °C for 20 min. Two μg of digested chromatin was diluted in a final volume of 0.8 ml containing 1% Triton X-100. Ligation reaction was carried out at 16 °C for 4 h and followed by incubation at room temperature for a further 30 min in the presence of T4 DNA ligase (New England BioLabs). Reverse cross-linking of ligated DNA was carried out overnight at 65 °C in the presence of proteinase K. The following day, samples were incubated for 30 min at 37 °C with RNase A (10 ng/ml) followed by purification and determination of DNA concentration. Nested primers were used with two rounds of PCR to increase sensitivity, which was followed by qPCR using a primer set that encompassed the BglII restriction cut site (Table 3) under the following conditions using the Power SYBR PCR kit (Applied Biosystems): the initial denaturation for 5 min at 95 °C, 30 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. The results were normalized to GAPDH in ligated and unligated samples. Interaction between HS I/II and the *hGH1* promoter (GHp) was determined by calculating the ligation frequency according to the formula [Ligation frequency = (level of ligation product/loading control) − (level of non-ligation product/loading control)].

Statistical Analysis—Statistical analysis was performed using GraphPad Prism® software. For the rhythmicity analysis and thus multiple comparisons between each time point, one-way ANOVA was used with the Bonferroni post-test. The diet effect at each time point (single comparisons) was assessed by paired Student's *t* test. A value of $p < 0.05$ is considered statistically significant and represented in figures as follows: *, $p < 0.05$; ** or ##, $p < 0.01$; *** or ###, $p < 0.001$.

Results

Human GH Unlike Mouse GH RNA Levels Oscillate over a 24-h Period—Anterior pituitary tissue was isolated every 4 h over a 26-h period from 4–5-week-old male hGH/CS-TG mice, fed a regular CD, and maintained under a 12-h light/dark cycle. Levels of *hGH1* RNA displayed a circadian pattern with high and comparable levels at ZT 2 and 26 (8 a.m.) that was significantly reduced by >65% at ZT 14 (12 p.m.) ($p < 0.001$, $n = 6$–12/time point) (Fig. 1*A*). By contrast, *mGh* RNA levels showed no significant oscillation over the same 26-h period (Fig. 1*B*).

Human GH Promoter E-box Supports Bmal1/Clock Heterodimer Binding—We showed previously that the *hGH1* promoter region contains a palindromic hexanucleotide (5′-CACGTG-3′) E-box element at position −264 to −259 (34). This E-box is located within the chromatin loop region linking *hGH1* promoter and HS I/II regions that is required for *hGH1* activation and efficient postnatal expression (26) but is not present in the same region of *mGh* sequences (34). Thus, EMSA was used to test the ability of this sequence to support binding

of the E-box factors Bmal1/Clock. Expression of Bmal1 and its partner Clock in transfected HEK293 cells was confirmed by electrophoresis of nuclear and cytosolic extracts and immunodetection (Fig. 2*A*). EMSA was done using nuclear extracts from HEK293 cells overexpressing Bmal1 and Clock proteins with radiolabeled *hGH1* promoter E-box element (nucleotides −278/−250) as a probe. Multiple low to high mobility complexes were seen with nuclear extract from HEK293 cells transfected with an empty vector (Fig. 2*B*). The intensity of these complexes and particularly the two large low mobility complexes (Fig. 2*B*, *arrows*) were increased with nuclear extract from HEK293 cells expressing Bmal1 and Clock cDNAs (Fig. 2*B*). The low and high mobility complexes were competed effectively with (50- and 100-fold) molar excess unlabeled WT *hGH1* promoter E-box element (5′-CACGTG-3′). By contrast, use of two oligonucleotides, Mut1 and Mut2, containing two (5′-CAgcTG-3′) and six (5′-gatcga-3′) base pair mutations of the WT *hGH1* promoter E-box element (45), respectively, were progressively less effective as competitors (Fig. 2*B*).

Bmal1/Clock Overexpression Transactivates a hGH1p-Luciferase Reporter Gene via Association with an E-box DNA Element—HEK293 cells were transiently transfected with hybrid luciferase genes driven by 496 bp of *hGH1* promoter sequences with a WT or mutated (Mut1) E-box DNA element; the latter was introduced by PCR-based site-directed mutagenesis. Both hybrid Luc genes were co-transfected with either control empty vector or a Bmal1/Clock expression vector. A significant increase in expression was detected in the presence of the WT ($p < 0.001$, $n = 9$), but not Mut1 E-box DNA element in response to Bmal1/Clock cDNA expression (Fig. 3*A*). A hybrid firefly luciferase reporter gene driven by equivalent (0.5 kb) *mGh* promoter sequences that contains no E-box DNA element was also assessed. No response to Bmal1/Clock cDNA expression was observed (Fig. 3*B*). A hybrid human Per2 promoter/firefly luciferase reporter gene, which contains three E-box DNA elements, was used as a positive control (39). As expected, the activity of the Per2 promoter was increased significantly by Bmal1/Clock protein cDNA expression ($p < 0.001$, $n = 6$) (Fig. 3*C*).

Rhythmicity Reflected in hGH RNA and Protein Levels Is Disrupted after 3 Days on a High Fat Diet—To assess the effect of acute high caloric intake on hGH production over a 24-h period, 4-week-old male hGH/CS-TG mice were fed either a CD or a HFD for 3 days, and pituitary RNA and protein was isolated every 4 h and assessed by qPCR and ELISA. The HFD caused a significant 45% reduction ($p < 0.01$, $n = 12$) in *hGH1* RNA levels at the time point with the highest levels detected on the CD, specifically during daylight hours ZT 0–2 (Fig. 4*A*). Serum hGH levels were also reduced throughout the study period in mice on the HFD compared with CD and specifically at ZT 2 and 22 (8 a.m. and 4 a.m.) ($p < 0.05$, $n = 5$–6) (Fig. 4*B*). By contrast, no significant changes were detected in *mGh* transcript levels over the 24-h period on either diet or differences between effects of a HFD *versus* CD at any time point (Fig. 4*C*). Like the hGH/CS-TG mice, there were also no detectable differences seen in mGH RNA levels in wild type mice fed the HFD *versus* CD for 3 days at any time point over the 24 h ($n = 4$) (Fig. 4*D*).
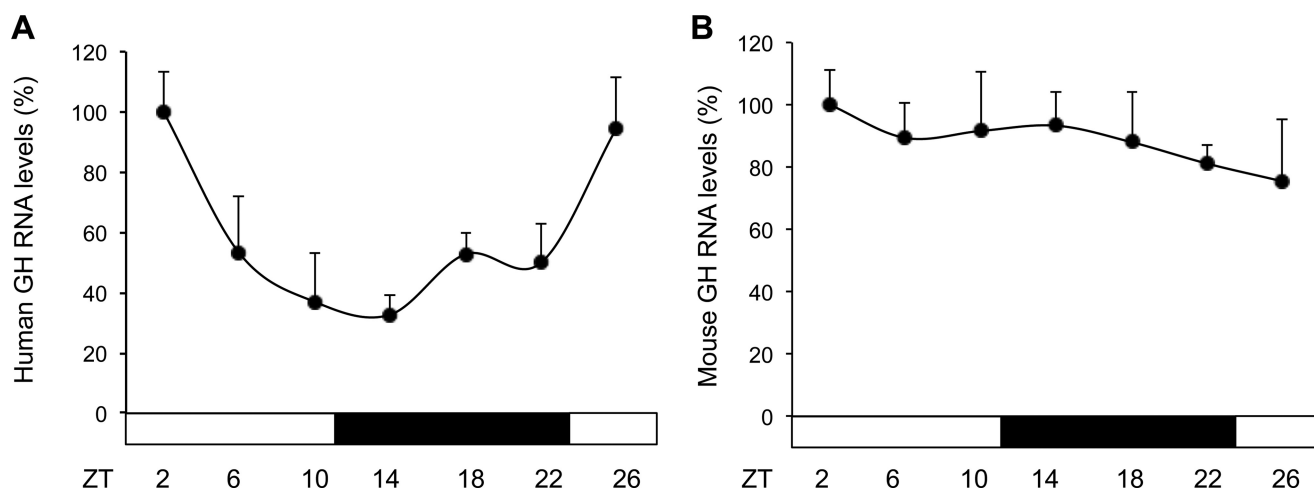
FIGURE 1. **Human GH RNA expression, unlike that of the mouse, oscillates over a 24-h period.** Anterior pituitary tissues were isolated every 4 h over a 24-h period from 4–5-week-old male mice, fed a regular CD, and maintained under a 12-h light/dark cycle. Total pituitary RNA from hGH/CS TG mice at each time point was assessed by qPCR for human GH (*A*) and mGH RNA (*B*) levels. The results are expressed as mean percentage change ± standard error of mean relative to levels detected at ZT 2, which is arbitrarily set to 100%, *n* = 6–12.
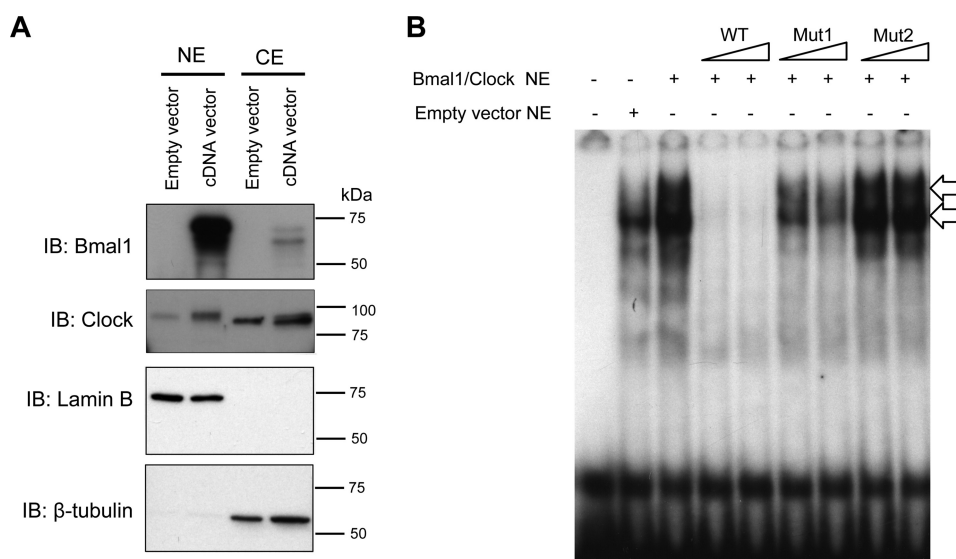

FIGURE 2. **Human GH promoter E-box supports Bmal1/Clock heterodimer binding.** *A*, expression of Bmal1 (~69 kDa) and its partner Clock (~95 kDa) in transfected HEK293 cells was confirmed by electrophoresis of nuclear (*NE*) and cytosolic (*CE*) extracts and immunodetection. β-Tubulin (~55 kDa) and lamin B (~66 kDa) were used as loading control for CE and NE proteins, respectively. *B*, EMSA was done using NE from HEK293 cells overexpressing Bmal1 and Clock proteins with radiolabeled *hGH1* promoter E-box element (nucleotides −278/−250) as a probe. The effects of molar excess (50- and 100-fold) of unlabeled WT *hGH1* promoter E-box element (with 5′-CACGTG-3′ core sequence) and two oligonucleotides, Mut1 and Mut2, containing two (5′-CAgcTG-3′) and six (5′-gatcga-3′) base pair mutations of the WT *hGH1* promoter E-box core sequence were used with their respective WT radiolabeled counterpart. Major low mobility DNA-protein complexes are indicated (*open arrowheads*). *IB*, immunoblotting.

The effect of excess caloric intake on core clock machinery gene diurnal expression, Clock, Bmal1, and Per2 RNA levels were also assessed. Clock RNA levels displayed a variation during the diurnal cycle with peaks during the onset of daylight hours (ZT 2) and troughs during dark hours (ZT 10–22). HFD consumption for 3 days led to a lack of diurnal variation in Clock RNA levels in the pituitary gland because a decreasing trend was detected during the light phase and an increasing trend was detected during the night cycle when compared with the CD (Fig. 5*A*). However, these differences at each time point did not reach statistical significance (*n* = 12). There was a clear diurnal variation of both Bmal1 and Per2 RNAs with a peak at ZT 2 and ZT 14, respectively, in both CD and HFD groups. The HFD increased Bmal1 RNA levels significantly on the HFD

($p < 0.05$, $n = 12$) at ZT 22 during the dark period (Fig. 5*B*) but had no significant impact on Per2 RNA levels (Fig. 5*C*). Rev-erbα transcript levels displayed diurnal variation in mice on either a CD or HFD, with a peak during the light period (highest levels at ZT 10) and low levels throughout the dark period (Fig. 5*D*). By contrast, the opposite time-dependent cycling was observed for Cry1 and Cry2 RNA levels, which are essential components of the negative limb of the circadian clock feedback loop (46). Diurnal variation in Cry1 RNA levels in the CD group was at its maximum during the dark period (highest levels at ZT 18), whereas Cry2 RNA levels peaked at ZT 10 (Fig. 5, *E* and *F*). The HFD was associated with increased amplitude of Cry1 and a phase shift in Cry2 RNA levels, because peak levels were delayed and
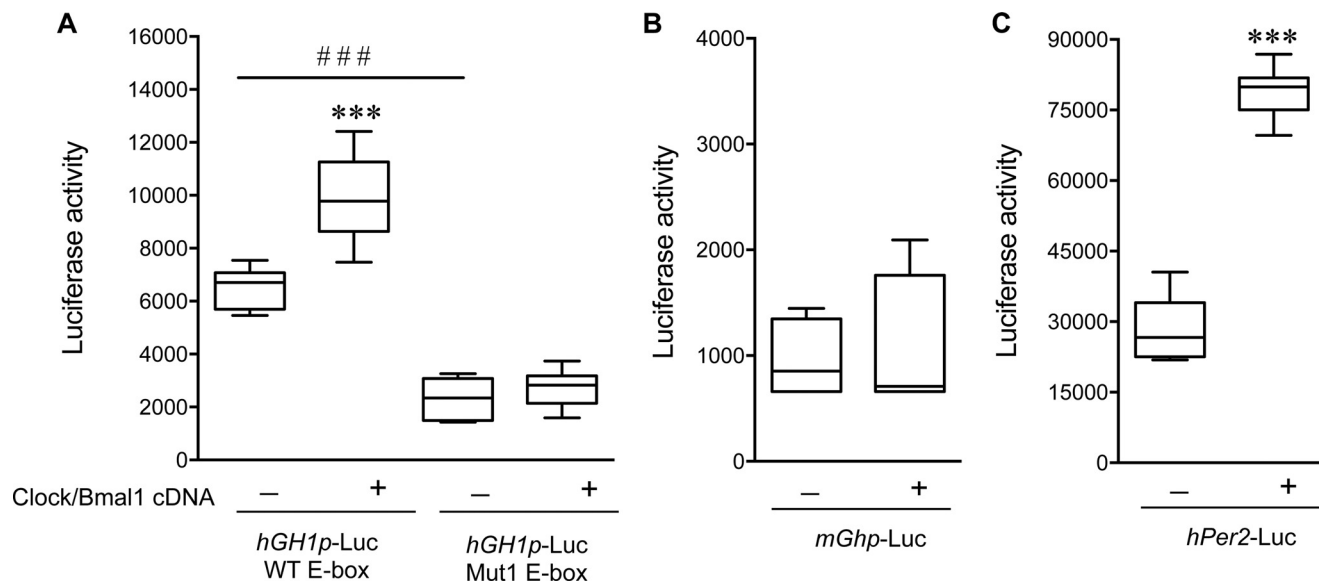
FIGURE 3. Bmal1/Clock overexpression in HEK293 cells transactivates hGH1p-luciferase reporter via association with an E-box DNA element. HEK293 cells were transiently transfected with hybrid luciferase (*Luc*) genes driven by: 496 bp of *hGH1* promoter sequences with a WT or Mut1 E-box DNA element (*A*), a 0.5-kb *mGh* promoter (*B*), and human Per2 promoter sequences (*C*). All hybrid Luc genes were co-transfected with either control empty vector or a Bmal1/Clock expression vector. The values were normalized through co-transfection with pRL-TKp-Luc and assessment of *Renilla* luciferase in a dual assay. The results are expressed in *box and whisker plots*. The *box limits* represent first and third quartiles, the *center line* represent the median, and the *whiskers* represent minimum and maximum values. Significant differences are indicated: *** and ###, $p < 0.001$, $n = 6$.
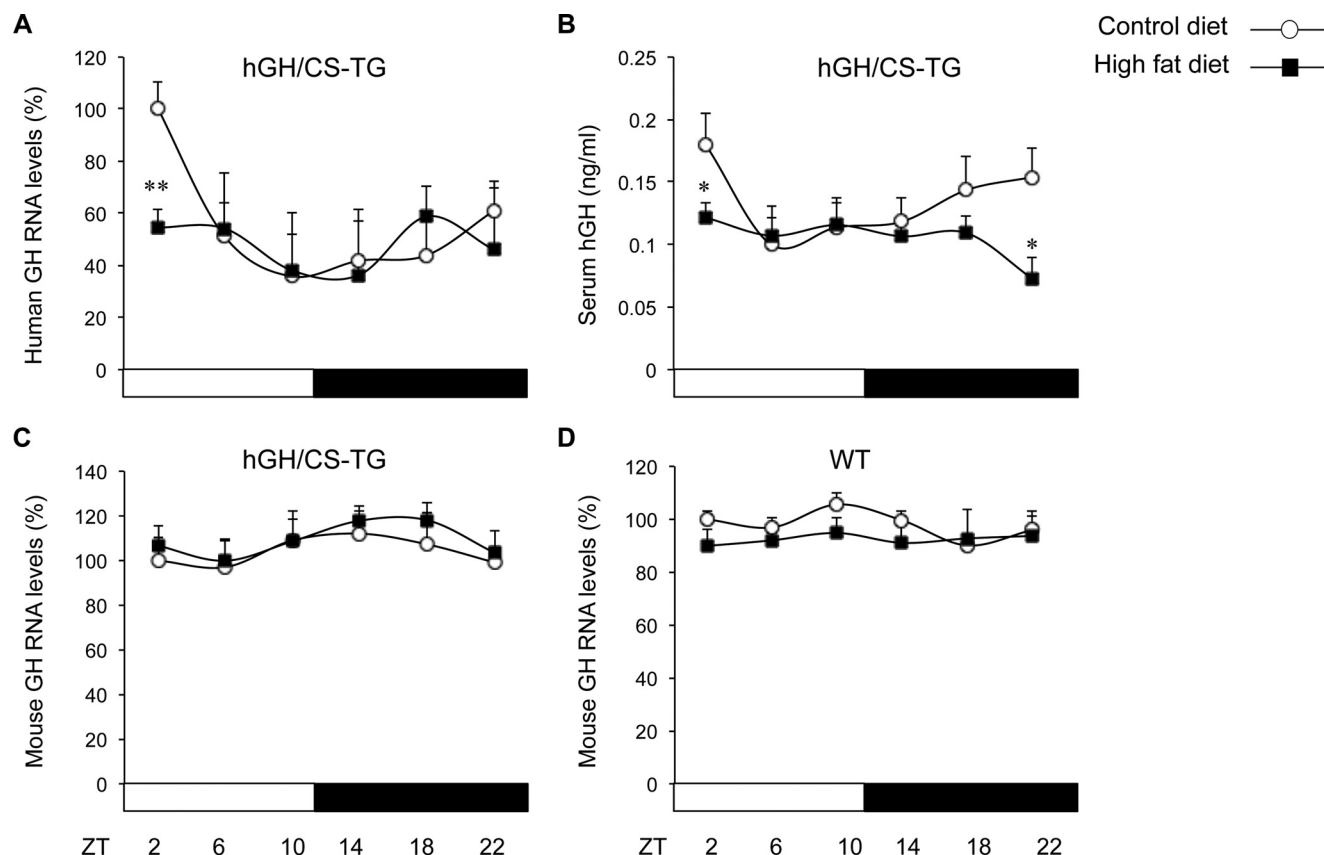

FIGURE 4. The effect of acute high caloric intake on rhythmic synthesis and secretion of hGH over a 24-h period. *A* and *B*, human pituitary GH RNA (*A*) and secreted protein (*B*) levels were assessed by qPCR and ELISA respectively every 4 h for 24 h, after 3 days on a control or high fat diet. *C* and *D*, the levels of mGH RNA in hGH/CS TG (*C*) and WT CD1 mice (*D*) at 3 days were also assessed as above. The results are expressed as mean percentage change/concentration (ng/ml) ± standard error of mean relative to levels detected at ZT 2, which is arbitrarily set to 100%, $n = 6$–12. Significant differences are indicated: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

appeared during the onset of the dark period (Fig. 5, *E* and *F*). A diurnal expression is described for the c-*fos* gene in the pituitary gland (47), and rhythmic expression of this gene is

sensitive to excess caloric intake (48). As expected, c-*fos* RNA levels were reduced (70%) at early and late stages of the dark period after 3 days on a HFD (data not shown).
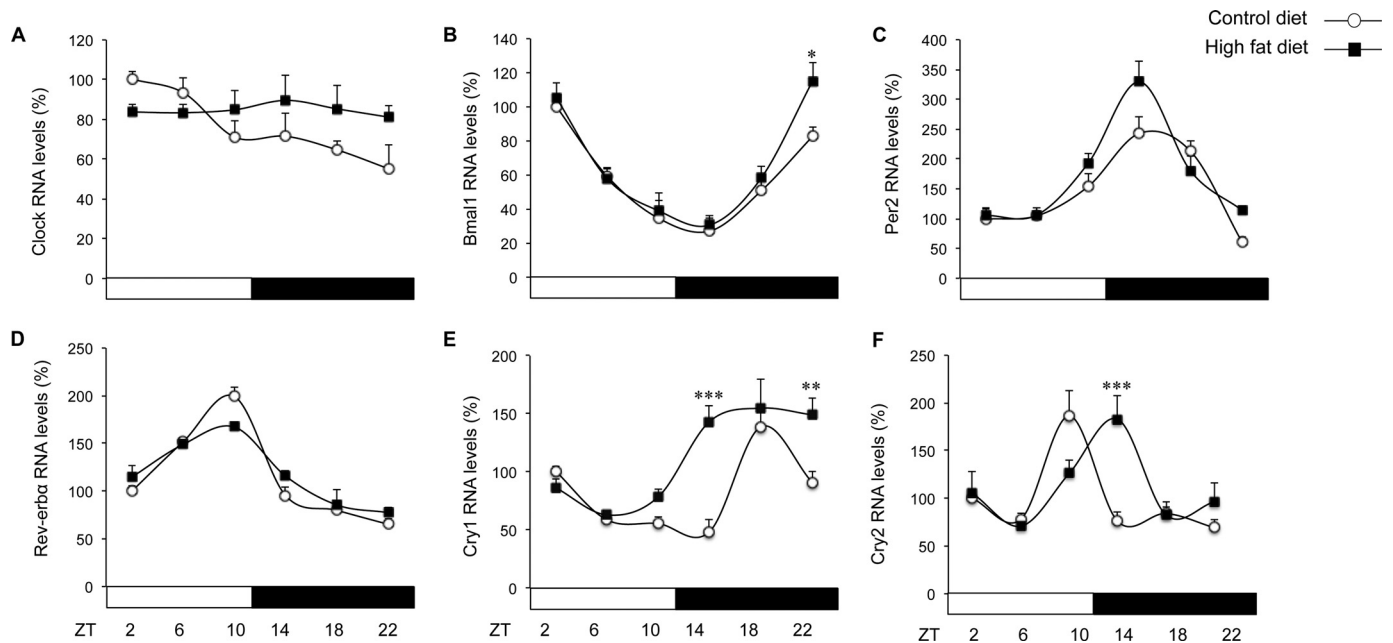
FIGURE 5. **The effect of acute high caloric intake on the rhythmicity of clock machinery RNA levels in the pituitary over a 24-h period.** RNA levels of the cellular clock machinery were assessed by qPCR every 4 h for 24 h, after 3 days on a control or high fat diet. The results are expressed as mean percentage change ± standard error of mean relative to levels detected at ZT 2, which is arbitrarily set to 100%, n = 6–12. *A*, Clock. *B*, Bmal1. *C*, Per2. *D*, Rev-erbα. *E*, Cry1. *F*, Cry2. Significant differences are indicated: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Bmal1 Association with the hGH1 Promoter Is Reduced by Excess Caloric Intake—The effect of a CD *versus* HFD for 3 days on binding of Bmal1 to the *hGH1* promoter region in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages was assessed by ChIP assay. Association of Bmal1 with the *hGH1* promoter region was reduced significantly at ZT 2 but was not decreased at ZT 18 for mice on the HFD *versus* CD (Fig. 6). Furthermore, Bmal1 levels for mice on the HFD were comparable and thus restored to those detected on the CD in the dark stage at ZT 18. However, a comparison of Bmal1 association with the *hGH1* promoter region at ZT 2 *versus* ZT 18 in mice fed a CD reveals a significant 30% reduction binding ($p < 0.01$, $n = 3$) in the dark stage (Fig. 6). No effect of diet was observed on Bmal1 association with an untranscribed region on mouse chromosome 6 (Untr6), which served as a measure of nonspecific association of DNA protein in mouse pituitary at either ZT 2 or 18 (Fig. 6).

A Chromatin Loop Required for Efficient Postnatal hGH1 Expression Is Negatively Affected by a HFD in the Light (Inactive) but Not Dark (Active) Phase of the Day in Mice—The effect of a CD *versus* HFD for 3 days on *hGH1* promoter and HS I/II region interactions (evidence of chromatin looping) in mouse pituitary during the light (ZT 2) and dark (ZT 18) phases was assessed by 3C assay (Fig. 7). A significant 65% decrease in ligation frequency of the *hGH1* promoter and HS I/II fragment was detected at ZT 2, the light and relatively inactive phase in mice ($p < 0.001$, $n = 3$). This decrease with a HFD was not apparent, however, by ZT 18 (dark and relatively active phase in mice), where levels were comparable with those of mice on a CD at ZT 18 ($p < 0.01$, $n = 3$). A reduction in the ligation frequency between the *hGH1* promoter and HS I/II regions was also suggested in the pituitaries of mice fed with CD at ZT 18 when compared with ZT 2 but did not reach statistical significance ($p = 0.07$, $n = 3$).
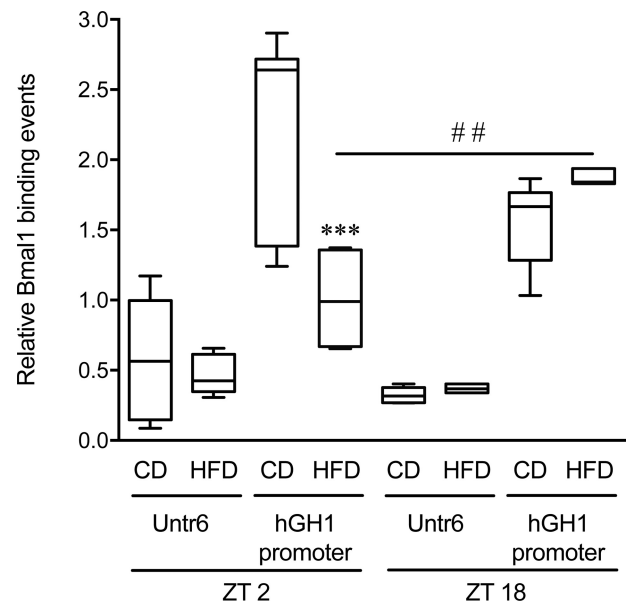

FIGURE 6. **Bmal1 association with the hGH1 promoter is reduced by excess caloric intake.** ChIP was performed with an anti-Bmal1 antibody to assess changes in association of Bmal1 with the *hGH1* promoter in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages in response to a CD *versus* a HFD for 3 days. Binding events were normalized to the amount of input chromatin used for the Bmal1 immunoprecipitation reaction and the control untranscribed regions of mouse (Untr6). The results are expressed in *box and whisker plots*. The *box limits* represent first and third quartiles, the *center line* represents the median, and the *whiskers* represent minimum and maximum values. Comparisons were made to the control value (control diet), which is arbitrarily set to 1.0. Significant differences were assessed by *t* test and are indicated: **, ## $p < 0.01$; and ***, ###, $p < 0.001$, $n = 3$.

Discussion

In this study, we show that *hGH1* but not *mGh* is expressed rhythmically and that RNA levels oscillate over a 24-h period in hGH/CS-TG mice. Analysis of *hGH1* proximal promoter
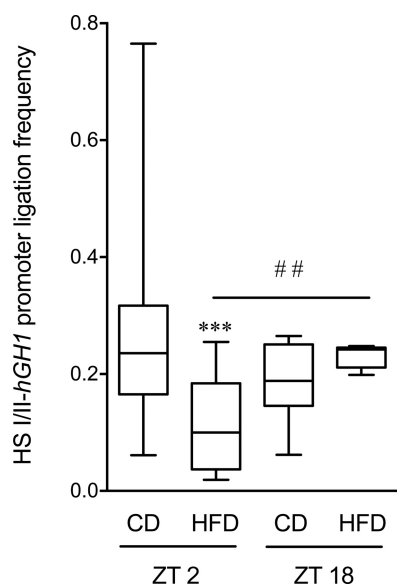
FIGURE 7. A chromatin loop required for efficient postnatal hGH1 expression is negatively affected by a HFD in the light (inactive) but not dark (active) stage of the day in mice. The effect of a CD *versus* a HFD for 3 days on *hGH1* promoter and HS I/II region interactions in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages was assessed by 3C assay. The results are normalized to GAPDH from ligated and unligated samples. Interaction between HS I/II and the *hGH1* promoter was determined by calculating the ligation frequency. The results are expressed in *box and whisker plots*. The *box limits* represent first and third quartiles, the *center line* represents the median, and the *whiskers* represent minimum and maximum values. Significance was assessed by t test, $p < 0.001$. Significance was assessed by t test: ##, $p < 0.01$; and ***, $p < 0.001$, $n = 3$.

sequences revealed the presence of an E-box element that was not present in equivalent *mGh* DNA (34). We provide evidence that these sequences have the capacity to bind the E-box factors Bmal1 and Clock and transactivate the *hGH1* promoter, using EMSA, ChIP, and transient transfection assays. Association of Bmal1 with the E-box element was reduced in male mice fed a HFD during the light stage of the 24-h period *in situ*. By contrast, this decrease was not seen during the dark stage, which is associated with a time of greater daily activity in mice. These changes also correlate with a significant negative impact of excess caloric intake on the *hGH1* chromosomal structure, involving interaction between remote enhancer and proximal promoter regions that is required for efficient postnatal expression and thus a negative impact on the hGH production.

Levels of *hGH1* RNA displayed a circadian pattern with high and comparable levels at ZT 2 and 26 (8 a.m.), which was significantly reduced at ZT 14 (12 p.m.) in *hGH1* transgenic mice. Thus, the *hGH1* transcript profile has the appearance of a classic clock-controlled gene with a peak and trough that are 12 h apart under light and dark conditions (49). By contrast, mGH RNA levels showed no significant oscillation over the same 26-h period. This difference might be related to the E-box element, with 5′-CACGTG-3′ at its core, located in the *hGH1* promoter region that is absent from equivalent *mGh* sequences (34). Efficient postnatal expression and even activation of *hGH1* is linked to pituitary specific transcription factor Pit-1 binding, at both the promoter and remotely 14.5 kb upstream at HS I/II within the *hGH1* LCR (50). These Pit-1 binding regions interact through looping out of intervening sequences (50). Evidence

presented here and in previous studies indicates that sequences within the chromatin loop, and specifically an E-box DNA element at nucleotide position −264/−259 (relative to the *hGH1* transcription initiation site), plays a role in maintaining basal promoter activity (33, 34, 51). Results from EMSA support the ability of Bmal1/Clock and *hGH1* E-box DNA element to participate in a common complex. Furthermore, mutation of two nucleotides at the core of the E-box element significantly impaired basal promoter activity, and deletion of nucleotides −266 to −253 that includes the E-box element completely suppressed *hGH1* promoter activity (52). Although the focus here has been on the potential contribution of an E-box element located at nucleotide position −264/−259, we cannot rule out the possibility that other E-box elements play a role in circadian oscillation of *hGH1*. However, location as in association with *hGH1* chromatin loop-related sequences may be as important as the simple presence of an E-box element(s), given the lack of response seen with *mGh*.

Excess caloric intake had a negative impact on *hGH1* RNA levels at only one time point during the day when the highest levels of the transcript were detected (ZT 2). This reduction at ZT 2, however, was associated with significant >40% decreases in hGH detected in the circulation at ZT 2 and 22 (8 a.m. and 4 a.m.), when the highest levels would normally be detected. Thus, our data are consistent with a negative impact of a HFD for 3 days on rhythmic hGH production during the inactive locomotor phase, considering the nocturnal nature of mice as opposed to humans. This observation is consistent with the spikes in GH levels in humans during the biological evening and night or sleep/inactive period (53).

The altered *hGH1* expression might be related to the reduction seen in Bmal1 association with the *hGH1* promoter region for mice on the HFD *versus* CD at ZT 2 rather than effects on Bmal1 production. This pattern of clock-controlled gene dysregulation by a HFD has been reported in the liver (31). The mechanism involved disruption of Bmal1 association with the chromatin *in situ* without any significant changes in Bmal1 RNA and/or protein levels (31). This is consistent with our observation that the positive limb of the core clock transcriptional machinery including Clock and Bmal1 were not affected significantly by HFD at most time points assessed. Although Bmal1 RNA levels were increased significantly at the late stage of the dark cycle (ZT 22), the HFD had a significant positive effect at this time on the negative arm of the core clock machinery including Cry1. These increases in Bmal1 and Cry1 transcript levels with a HFD have been reported previously (54) and may reflect a disrupted feedback system. Evidence suggests that a physical interaction between Cry1 and Bmal1 is responsible for switching Bmal1 from an activated to a repressive state (55). Thus, earlier appearance of the Cry1 in the pituitaries of *hGH1* TG mice on the HFD may lead to repression of the Bmal1-Clock protein complex, which in turn signals an increase in Bmal1 production and increased RNA levels. A positive trend in Clock expression during the dark cycle by HFD was also observed, although it did not reach statistical significance (55).

It is noted that Bmal1-Clock complexes have significantly different chromatin binding properties at different times during the day, possibly because of post-translational modifica-

tion (56). Thus, binding of these transcription factors to E-box elements follows a circadian pattern *in vivo* (57). The Bmal1 ChIP analysis supports this notion; there was a difference between the levels of Bmal1 associated with the *hGH1* promoter region based on the time of euthanization (light *versus* dark stage). Because the Bmal1-Clock complex does not remain bound to chromatin throughout the day (58), the opportunity for other E-box-associated factors to bind and perhaps modulate promoter activity is possible. As a result, a circadian exchange of different transcription factors binding to E-box elements would offer a target for a variety of signaling pathways, perhaps reflecting environmental and/or intrinsic cellular cues to influence transcriptional activity depending on physiological demand.

We have previously shown that the hGH gene (*hGH1*) promoter region is hypoacetylated in response to excess caloric intake at time points between ZT 2 and 6 (33). Histone modifications are implicated in the transcriptional regulation of the *hGH1* locus. Thus, the *hGH1* promoter region is condensed and can limit the activator transcription factor such as Bmal1-Clock heterodimer accessibility. Furthermore, transcriptional repression is mediated by recruitment of co-repressor complexes, which can lead to chromatin remodeling by directing histone modification enzymes to specific site of regulatory elements of the target gene. Among the most well characterized transcriptional co-repressors of the pituitary gland is the nuclear receptor co-repressor. We have previously demonstrated that the nuclear receptor co-repressor can mediate the transcriptional repression of *hGH1* in somatotrophs/pituitary from 171hGH/CS TG mice overfed for 3 days (33). Excess caloric intake resulted in a 4-fold increase in association of the nuclear receptor co-repressor with the *hGH1* promoter region in the pituitary as assessed by ChIP assay (33).

A recent study implicated the circadian machinery in determining temporal and spatial cycles of chromosomal organization and is capable of affecting the physical organization of the nuclear landscape via involvement of E-box elements (59). The diurnal variation of *hGH1* transcript levels raises the possibility that the *hGH1* locus can transit between an active and inactive chromosomal conformation in a cyclic manner daily. If so, the physical interaction between HS I/II and the proximal promoter chromatin regions may respond to or influence association of E-box transcription factor(s), as seen with Bmal1 and the *hGH1* promoter. Intriguingly, the reduction in the HS I/II-*hGH1* promoter chromatin loop observed during the light cycle with the HFD regimen was not evident during the dark stage of the cycle, suggesting a recovered and/or intact chromosomal conformation. This observation may reflect an example of transcriptional memory (60). It is anticipated that changes in the availability and accessibility of E-box factors would reverse the reduction in *hGH1* RNA levels and associated changes in the *hGH1* locus (chromatin remodeling). This would likely involve elimination of repressive factors resulting from HFD consumption during the dark stage (33).

A study of hGH synthesis is not possible in humans, but our studies in TG mice implicate the pituitary as a target for circadian control of *hGH1* transcription. To our knowledge, this provides the first evidence for rhythmic *hGH1* RNA levels that

correlate with the rhythmic hGH secretion reported in humans over a 24-h cycle. Our data also indicate that this rhythmic *hGH1* expression is sensitive to excess caloric intake that can disrupt association of the circadian machinery with the locus.

Author Contributions—H. V. and P. A. C. conceived and coordinated the study and wrote the paper. H. V. designed, performed, and analyzed the experiments. Y. J. performed the experiments shown in Figs. 6 and 7.

References

1. Andrews, R. V., and Folk, G. E., Jr. (1964) Circadian metabolic patterns in cultured hamster adrenal glands. *Comp. Biochem. Physiol.* **11,** 393–409
2. Takahashi, J. S., Hong, H. K., Ko, C. H., and McDearmon, E. L. (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat. Rev. Genet.* **9,** 764–775
3. Linkowski, P., Kerkhofs, M., Van Onderbergen, A., Hubain, P., Copinschi, G., L'Hermite-Balériaux, M., Leclercq, R., Brasseur, M., Mendlewicz, J., and Van Cauter, E. (1994) The 24-hour profiles of cortisol, prolactin, and growth hormone secretion in mania. *Arch. Gen. Psychiatry* **51,** 616–624
4. Linkowski, P., Mendlewicz, J., Kerkhofs, M., Leclercq, R., Golstein, J., Brasseur, M., Copinschi, G., and Van Cauter, E. (1987) 24-hour profiles of adrenocorticotropin, cortisol, and growth hormone in major depressive illness: effect of antidepressant treatment. *J. Clin. Endocrinol. Metab.* **65,** 141–152
5. Brandenberger, G., and Weibel, L. (2004) The 24-h growth hormone rhythm in men: sleep and circadian influences questioned. *J. Sleep Res.* **13,** 251–255
6. Weibel, L., Follenius, M., Spiegel, K., Gronfier, C., and Brandenberger, G. (1997) Growth hormone secretion in night workers. *Chronobiol. Int.* **14,** 49–60
7. Avram, A. M., Jaffe, C. A., Symons, K. V., and Barkan, A. L. (2005) Endogenous circulating ghrelin does not mediate growth hormone rhythmicity or response to fasting. *J. Clin. Endocrinol. Metab.* **90,** 2982–2987
8. Strasburger, C. J. (1990) Antigenic epitope mapping of the human growth hormone molecule: a strategy to standardize growth hormone immunoassays. *Acta Paediatr. Scand. Suppl.* **370,** 82–86
9. Yi, S., Bernat, B., Pál, G., Kossiakoff, A., and Li, W. H. (2002) Functional promiscuity of squirrel monkey growth hormone receptor toward both primate and nonprimate growth hormones. *Mol. Biol. Evol.* **19,** 1083–1092
10. Nickel, B. E., Kardami, E., and Cattini, P. A. (1990) The human placental growth hormone variant is mitogenic for rat lymphoma Nb2 cells. *Endocrinology* **126,** 971–976
11. Wells, J. A., Cunningham, B. C., Fuh, G., Lowman, H. B., Bass, S. H., Mulkerrin, M. G., Ultsch, M., and deVos, A. M. (1993) The molecular basis for growth hormone-receptor interactions. *Recent Prog. Horm. Res.* **48,** 253–275
12. Wells, J. A., and de Vos, A. M. (1993) Structure and function of human growth hormone: implications for the hematopoietins. *Annu. Rev. Biophys. Biomol. Struct.* **22,** 329–351
13. Jones, B. K., Monks, B. R., Liebhaber, S. A., and Cooke, N. E. (1995) The human growth hormone gene is regulated by a multicomponent locus control region. *Mol. Cell. Biol.* **15,** 7010–7021
14. Lira, S. A., Kalla, K. A., Glass, C. K., Drolet, D. W., and Rosenfeld, M. G. (1993) Synergistic interactions between Pit-1 and other elements are required for effective somatotroph rat growth hormone gene expression in transgenic mice. *Mol. Endocrinol.* **7,** 694–701
15. Jin, Y., Lu, S. Y., Fresnoza, A., Detillieux, K. A., Duckworth, M. L., and Cattini, P. A. (2009) Differential placental hormone gene expression dur-

ing pregnancy in a transgenic mouse containing the human growth hormone/chorionic somatomammotropin locus. *Placenta* **30,** 226–235

16. Vakili, H., Jin, Y., Nagy, J. I., and Cattini, P. A. (2011) Transgenic mice expressing the human growth hormone gene provide a model system to study human growth hormone synthesis and secretion in non-tumor-derived pituitary cells: differential effects of dexamethasone and thyroid hormone. *Mol. Cell. Endocrinol.* **345,** 48–57

17. Grosveld, F., van Assendelft, G. B., Greaves, D. R., and Kollias, G. (1987) Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell* **51,** 975–985

18. Elefant, F., Cooke, N. E., and Liebhaber, S. A. (2000) Targeted recruitment of histone acetyltransferase activity to a locus control region. *J. Biol. Chem.* **275,** 13827–13834

19. Jin, Y., Surabhi, R. M., Fresnoza, A., Lytras, A., and Cattini, P. A. (1999) A role for A/T-rich sequences and Pit-1/GHF-1 in a distal enhancer located in the human growth hormone locus control region with preferential pituitary activity in culture and transgenic mice. *Mol. Endocrinol.* **13,** 1249–1266

20. Bennani-Baïti, I. M., Asa, S. L., Song, D., Iratni, R., Liebhaber, S. A., and Cooke, N. E. (1998) DNase I-hypersensitive sites I and II of the human growth hormone locus control region are a major developmental activator of somatotrope gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10655–10660

21. Fleetwood, M. R., Ho, Y., Cooke, N. E., and Liebhaber, S. A. (2012) DNase I hypersensitive site II of the human growth hormone locus control region mediates an essential and distinct long-range enhancer function. *J. Biol. Chem.* **287,** 25454–25465

22. Su, Y., Liebhaber, S. A., and Cooke, N. E. (2000) The human growth hormone gene cluster locus control region supports position-independent pituitary- and placenta-specific expression in the transgenic mouse. *J. Biol. Chem.* **275,** 7902–7909

23. Ho, Y., Elefant, F., Cooke, N., and Liebhaber, S. (2002) A defined locus control region determinant links chromatin domain acetylation with long-range gene activation. *Mol. Cell* **9,** 291–302

24. Ho, Y., Tadevosyan, A., Liebhaber, S. A., and Cooke, N. E. (2008) The juxtaposition of a promoter with a locus control region transcriptional domain activates gene expression. *EMBO Rep.* **9,** 891–898

25. Ho, Y., Elefant, F., Liebhaber, S. A., and Cooke, N. E. (2006) Locus control region transcription plays an active role in long-range gene activation. *Mol. Cell* **23,** 365–375

26. Ho, Y., Shewchuk, B. M., Liebhaber, S. A., and Cooke, N. E. (2013) Distinct chromatin configurations regulate the initiation and the maintenance of hGH gene expression. *Mol. Cell. Biol.* **33,** 1723–1734

27. Bozek, K., Relógio, A., Kielbasa, S. M., Heine, M., Dame, C., Kramer, A., and Herzel, H. (2009) Regulation of clock-controlled genes in mammals. *PLoS One* **4,** e4882

28. Hogenesch, J. B., Gu, Y. Z., Jain, S., and Bradfield, C. A. (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5474–5479

29. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280,** 1564–1569

30. Duez, H., and Staels, B. (2010) Nuclear receptors linking circadian rhythms and cardiometabolic control. *Arterioscler. Thromb. Vasc. Biol.* **30,** 1529–1534

31. Eckel-Mahan, K. L., Patel, V. R., de Mateo, S., Orozco-Solis, R., Ceglia, N. J., Sahar, S., Dilag-Penilla, S. A., Dyar, K. A., Baldi, P., and Sassone-Corsi, P. (2013) Reprogramming of the circadian clock by nutritional challenge. *Cell* **155,** 1464–1478

32. Cornford, A. S., Barkan, A. L., and Horowitz, J. F. (2011) Rapid suppression of growth hormone concentration by overeating: potential mediation by hyperinsulinemia. *J. Clin. Endocrinol. Metab.* **96,** 824–830

33. Vakili, H., Jin, Y., and Cattini, P. A. (2014) Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus. *J. Clin. Invest.* **124,** 5002–5012

34. Vakili, H., Jin, Y., and Cattini, P. A. (2012) Negative regulation of human growth hormone gene expression by insulin is dependent on hypoxia-inducible factor binding in primary non-tumor pituitary cells. *J. Biol. Chem.* **287,** 33282–33292

35. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74

36. Nordeen, S. K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* **6,** 454–458

37. Jin, Y., Oomah, K., and Cattini, P. A. (2011) Enhancer-blocking activity is associated with hypersensitive site V sequences in the human growth hormone locus control region. *DNA Cell Biol.* **30,** 995–1005

38. Langmesser, S., Tallone, T., Bordon, A., Rusconi, S., and Albrecht, U. (2008) Interaction of circadian clock proteins PER2 and CRY with BMAL1 and CLOCK. *BMC Mol. Biol.* **9,** 41

39. Xu, Y., Toh, K. L., Jones, C. R., Shin, J. Y., Fu, Y. H., and Ptácek, L. J. (2007) Modeling of a human circadian mutation yields insights into clock regulation by PER2. *Cell* **128,** 59–70

40. Aza-Carmona, M., Shears, D. J., Yuste-Checa, P., Barca-Tierno, V., Hisado-Oliva, A., Belinchón, A., Benito-Sanz, S., Rodríguez, J. I., Argente, J., Campos-Barros, A., Scambler, P. J., and Heath, K. E. (2011) SHOX interacts with the chondrogenic transcription factors SOX5 and SOX6 to activate the aggrecan enhancer. *Hum. Mol. Genet.* **20,** 1547–1559

41. Norquay, L. D., Yang, X., Jin, Y., Detillieux, K. A., and Cattini, P. A. (2006) Hepatocyte nuclear factor-3α binding at P sequences of the human growth hormone locus is associated with pituitary repressor function. *Mol. Endocrinol.* **20,** 598–607

42. Yochum, G. S., McWeeney, S., Rajaraman, V., Cleland, R., Peters, S., and Goodman, R. H. (2007) Serial analysis of chromatin occupancy identifies β-catenin target genes in colorectal carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 3324–3329

43. Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002) Capturing chromosome conformation. *Science* **295,** 1306–1311

44. Miele, A., and Dekker, J. (2009) Mapping cis- and trans-chromatin interaction networks using chromosome conformation capture (3C). *Methods Mol. Biol.* **464,** 105–121

45. Arpiainen, S., Lämsä, V., Pelkonen, O., Yim, S. H., Gonzalez, F. J., and Hakkola, J. (2007) Aryl hydrocarbon receptor nuclear translocator and upstream stimulatory factor regulate cytochrome P450 2a5 transcription through a common E-box site. *J. Mol. Biol.* **369,** 640–652

46. Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., and Reppert, S. M. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98,** 193–205

47. Girotti, M., Weinberg, M. S., and Spencer, R. L. (2007) Differential responses of hypothalamus-pituitary-adrenal axis immediate early genes to corticosterone and circadian drive. *Endocrinology* **148,** 2542–2552

48. Mendoza, J., Pévet, P., and Challet, E. (2008) High-fat feeding alters the clock synchronization to light. *J. Physiol.* **586,** 5901–5910

49. Gamble, K. L., Berry, R., Frank, S. J., and Young, M. E. (2014) Circadian clock control of endocrine factors. *Nat. Rev. Endocrinol.* **10,** 466–475

50. Shewchuk, B. M., Asa, S. L., Cooke, N. E., and Liebhaber, S. A. (1999) Pit-1 binding sites at the somatotrope-specific DNase I hypersensitive sites I, II of the human growth hormone locus control region are essential for in vivo hGH-N gene activation. *J. Biol. Chem.* **274,** 35725–35733

51. Lemaigre, F. P., Courtois, S. J., Lafontaine, D. A., and Rousseau, G. G. (1989) Evidence that the upstream stimulatory factor and the Sp1 transcription factor bind *in vitro* to the promoter of the human-growth-hormone gene. *Eur. J. Biochem.* **181,** 555–561

52. Courtois, S. J., Lafontaine, D. A., and Rousseau, G. G. (1992) Characterization of an alternative promoter in the human growth hormone gene. *J. Biol. Chem.* **267,** 19736–19743

53. Uchiyama, M., Ishibashi, K., Enomoto, T., Nakajima, T., Shibui, K., Hirokawa, G., and Okawa, M. (1998) Twenty-four hour profiles of four hormones under constant routine. *Psychiatry Clin. Neurosci.* **52,** 241–243

54. Duivenvoorde, L. P., van Schothorst, E. M., Bunschoten, A., and Keijer, J. (2011) Dietary restriction of mice on a high-fat diet induces substrate efficiency and improves metabolic health. *J. Mol. Endocrinol.* **47,** 81–97

55. Xu, H., Gustafson, C. L., Sammons, P. J., Khan, S. K., Parsley, N. C., Ramanathan, C., Lee, H. W., Liu, A. C., and Partch, C. L. (2015) Crypto-

chrome 1 regulates the circadian clock through dynamic interactions with the BMAL1 C terminus. *Nat. Struct. Mol. Biol.* **22,** 476–484

56. Gallego, M., and Virshup, D. M. (2007) Post-translational modifications regulate the ticking of the circadian clock. *Nat. Rev. Mol. Cell Biol.* **8,** 139–148

57. Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107,** 855–867

58. Ripperger, J. A., and Schibler, U. (2006) Rhythmic CLOCK-BMAL1 bind-
ing to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat. Genet.* **38,** 369–374

59. Aguilar-Arnal, L., Hakim, O., Patel, V. R., Baldi, P., Hager, G. L., and Sassone-Corsi, P. (2013) Cycles in spatial and temporal chromosomal organization driven by the circadian clock. *Nat. Struct. Mol. Biol.* **20,** 1206–1213

60. Deng, W., and Blobel, G. A. (2010) Do chromatin loops provide epigenetic gene expression states? *Curr. Opin. Genet. Dev.* **20,** 548–554